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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of the project is to appraise critically the state of dietary prevention of breast cancer and to forge new avenues of investigation in the field of nutrition. A special emphasis is on the role of diet during fetal life, puberty, and pregnancy, in influencing breast development and breast cancer risk (task-1). In addition, the data we obtain using animal models serve as a basis of developing and conducting studies in human populations (task-3). Dietary factors that are the focus of these studies are fats, particularly polyunsaturated fatty acids (PUFAs), and phytoestrogens. Special emphasis is put on identifying their mechanism of action (task-2). In particular, the role of ER-α and ER-β and eicosanoids in mediating the effects of PUFAs and phytoestrogens are assessed. We will also determine whether BRCA1 is involved. In pubertal girls and adult parous women, estrogen-regulated factors, including epidermal growth factor, transforming growth factor α and insulin like growth factors, are studied in the serum and nipple aspirate fluid. During the funding period, a course is developed and directed at the Georgetown University as a new initiative to the existing Tumor Biology program, addressing critical nutritional issues in breast cancer (task-4).

Significant progress has been made towards all the four tasks, and this progress is detailed in this report. For example, we have shown that prepubertal estrogen exposure reduces breast cancer risk in an animal model, and our cohort study in 3,447 Finnish women support this conclusion. Further, our data indicate that up-regulation of ER-β protects the mammary gland from malignant transformation. Development of the proposed course has also been completed, and it will be offered to students starting in Spring 2001.

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INTRODUCTION

The purpose of Academic Award is to allow me to appraise critically the state of **dietary prevention of breast cancer** and to forge new avenues of investigation in the field of nutrition. These new avenues are achieved through studies that examine the role of diet during fetal life, puberty, and pregnancy, in influencing breast development and breast cancer risk. In addition, the data we obtain using animal models serve as a basis of developing and conducting studies in human populations. Dietary factors that are the focus of these studies are **fats**, particularly **polyunsaturated fatty acids,** and **phytoestrogens**. Special emphasis is put on identifying their mechanism of action. In particular, the role of the two estrogen receptor isotypes (ERα and ERβ) and eicosanoid pathways (cyclooxygenase, lipooxygenase and P450) in mediating the effects of PUFA and phytoestrogens are assessed. We also will determine whether BRCA1 is involved. In pubertal girls and adult parous women, estrogen-regulated factors, including epidermal growth factor, transforming growth factor α and insulin like growth factors, are studied in the serum and nipple aspirate fluid (NAF). During the funding period, a course is developed and directed at the Georgetown University as a new initiative to the existing Tumor Biology program, addressing critical nutritional issues in breast cancer.

BODY

Task-1. Effect of dietary manipulations occurring during sensitive periods, on breast cancer risk using animal models (months 1-24).

These dietary manipulations will occur during pregnancy and they include:

1.1. n-6 and n-3 PUFA

1.2. phytoestrogens

1.3. alcohol

Research accomplished associated with Task-1

We have presented a hypothesis that estrogens might play a dual role in affecting breast cancer risk [1]. Estrogens are generally associated with promotion of the growth of existing malignancies in the breast. However, these hormones and their metabolic products are also shown to induce direct and indirect free radical-mediated DNA damage, genetic instability and mutations in cells in culture and *in vivo*, suggesting a role for estrogens in cancer initiation. Further, estrogens may serve as pre-initiators. For example, elevated fetal estrogen levels can permanently alter the morphology of the mammary gland, and cause a persistent presence of epithelial structures (terminal end buds) that are known to be the sites of malignant growth. Data obtained in animal models and indirect evidence in humans indicate that high *in utero* estrogenicity increases breast cancer risk.

In contrast to these adverse effects of estrogens on the breast, in certain circumstances, such as during pregnancy that occurs prior age 20, estrogens actually reduce breast cancer risk. Our data obtained in animal models [2,3] and human populations [4] suggest that estrogenicty during childhood also reduces the risk. The reduction in breast cancer risk might occur through elimination of terminal end buds by differentiation. In addition, estrogen-induced activation of certain tumor suppressor genes, such as BRCA1 and p53 that are critical in DNA damage repair and in maintaining genetic stability, might reduce the likelihood that breast cancer will be initiated [1].

These findings raise the question as to whether timing of dietary exposures, particularly those that affect circulating estrogens, have varying effects on breast cancer risk. Therefore, we have studied the effects of a maternal exposure during pregnancy to (i) a high n-6 or n-3 polyunsaturated fatty acid

(PUFA) diet that increases pregnancy estradiol levels, (ii) a soy diet that contains phytoestrogen genistein, or (iii) genistein on offspring's mammary tumorigenesis. The results of these studies are summarized below.

1.1. n-6 and n-3 PUFA

***In utero* exposure.** We have earlier studied the effect of a maternal exposure during pregnancy to diets high or low in n-6 PUFAs [5]. The source of n-6 PUFA was corn oil, and an exposure to high n-6 PUFA diet significantly increased circulating estradiol levels in pregnant rats [5,6]. The results further indicated that a high maternal n-6 PUFA diet increases offspring's mammary. The carcinogen used to induce tumors was 7,12-dimethylbenz[a]anthracene (DMBA), and it was administered to offspring as a single oral 10 mg dose.

At present time, we are investigating whether a maternal intake of diets containing high or low levels of n-3 PUFAs affects mammary tumorigenesis. Menhaden oil is used as a dietary source of n-3 PUFA. The results show that pregnant rats consuming n-3 PUFAs, either in high or low fat diet, have significantly elevated circulating estrogen levels, compared to pregnant rats consuming high n-6 PUFA corn oil diet **(Fig. 1)**. Thus, if a diet contains menhaden oil, regardless whether the overall fat content is low or high, pregnancy estradiol levels are increased. The effect of n-3 PUFA diets on mammary tumorigenesis is currently being assessed, and therefore the data are not available yet.

1.2. Phytoestrogens

In utero exposures. We also have studied the effect of a maternal exposure during pregnancy to diets high or low in soy [7], or injected pregnant rats with genistein [8]. Genistein is a phytoestrogen and the component believed to mediate soy's effects on the breast; however, it binds and activates the estrogen receptor (ER) and therefore stimulates the growth of normal and malignant mammary cells both *in vitro* and *in vivo*.

Our earlier results indicate that *in utero* exposure to genistein dose-dependently increases offspring's

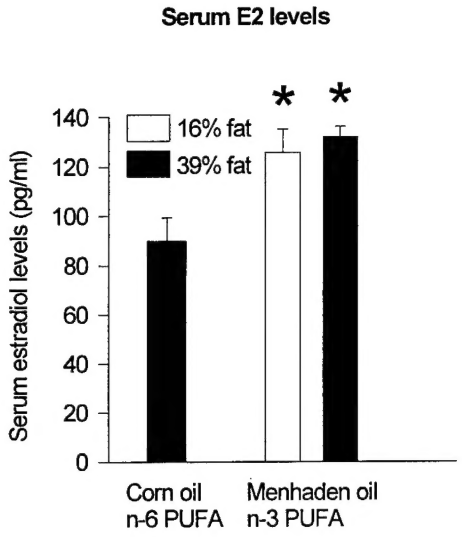


Fig. 1. Serum estradiol (E2) levels in pregnant rats on gestation day 19 who were fed diets containing low (16% energy) or high (39% energy) levels of fat. The fat source was either con oil or menhaden oil. The data indicate that menhaden oil, regardless of overall fat content, increases serum E2 levels, compared to corn oil. Significantly different compared with corn oil group:
* $p<0.05$

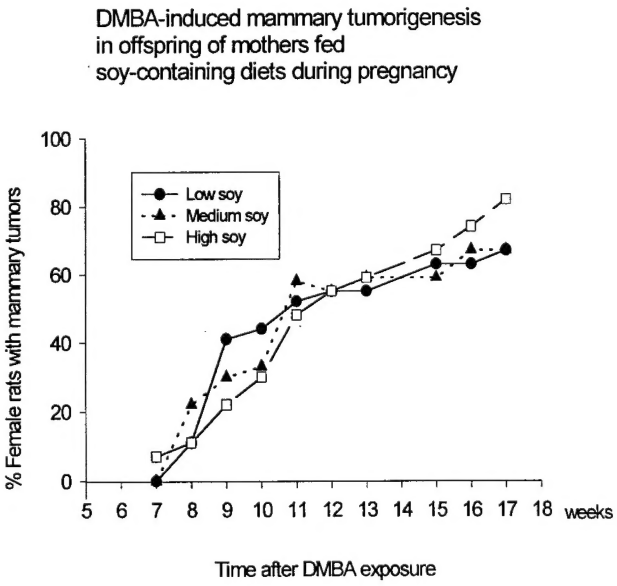


Fig. 2. Maternal exposure to medium or high soy diet during pregnancy does not affect DMBA-induced mammary tumorigenesis among female offspring.

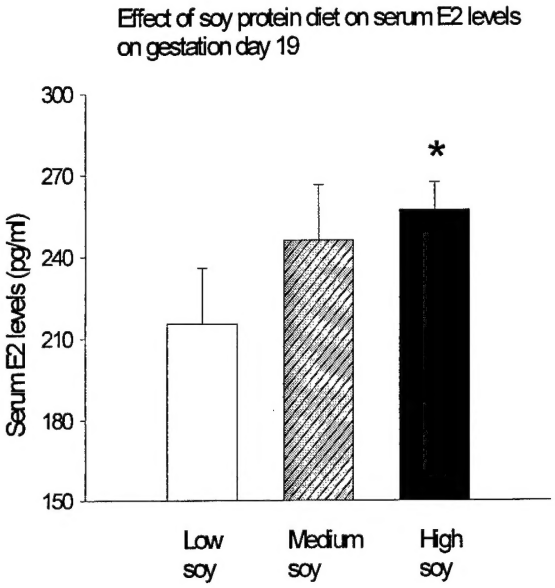


Fig. 3. Maternal exposure to high soy diet increases serum E2 levels during pregnancy. Significantly different from low soy fed rats: * $p<.0.05$.

mammary tumorigenesis [8]. Our recent data, however, show that maternal soy intake did not increase offspring's risk of developing mammary tumors **(Fig. 2)** [7], although the soy diet provided genistein at a comparable level to that when the pregnant rats were given genistein alone. The soy diet also increased pregnancy estrogen levels **(Fig. 3)**. These results indicate that soy must contain some additional components, which when administered *in utero*, reverse the effects of genistein on offspring's breast cancer risk.

Prepubertal exposures. Our group [3] has been studying the effects of prepubertal exposure to genistein on DMBA-induced mammary tumorigenesis. Further, the effects of prepubertal exposure to another phytoestrogen zearalenone, present as a contaminant in grains, corn, potato, rice and other similar farm products, and which effectively activates the ER, have been studied. Exposure to either of these two phytoestrogens between postnatal days 7 and 20 effectively reduced carcinogen-induced mammary tumor incidence.

We will next be studying whether an exposure to soy during prepubertal period has a similar risk reducing effect than exposure to genistein on mammary tumorigenesis.

1.3. Alcohol

Alcohol is known to increase serum estrogen levels, both following an acute administration and when exposed chronically. In collaboration with Dr. Richard Stevens (Department of Community Medicine, University of Connecticut Health Center, CT, USA), we are studying the impact of maternal alcohol exposure during pregnancy on offspring's breast cancer risk in a rat model. Preliminary studies to identify an appropriate maternal alcohol dose have already been performed, and a complete study will be done within the next 12 months.

Task-2 . Identification of mechanistic pathways that mediate the effects of PUFA, genistein, and alcohol on breast cancer risk (months 12-36).

These intermediate biomarkers include:

2.1. Eicosanoids, particularly Cox-2

2.2. ER-α and ER-β

Research accomplished associated with Task-2

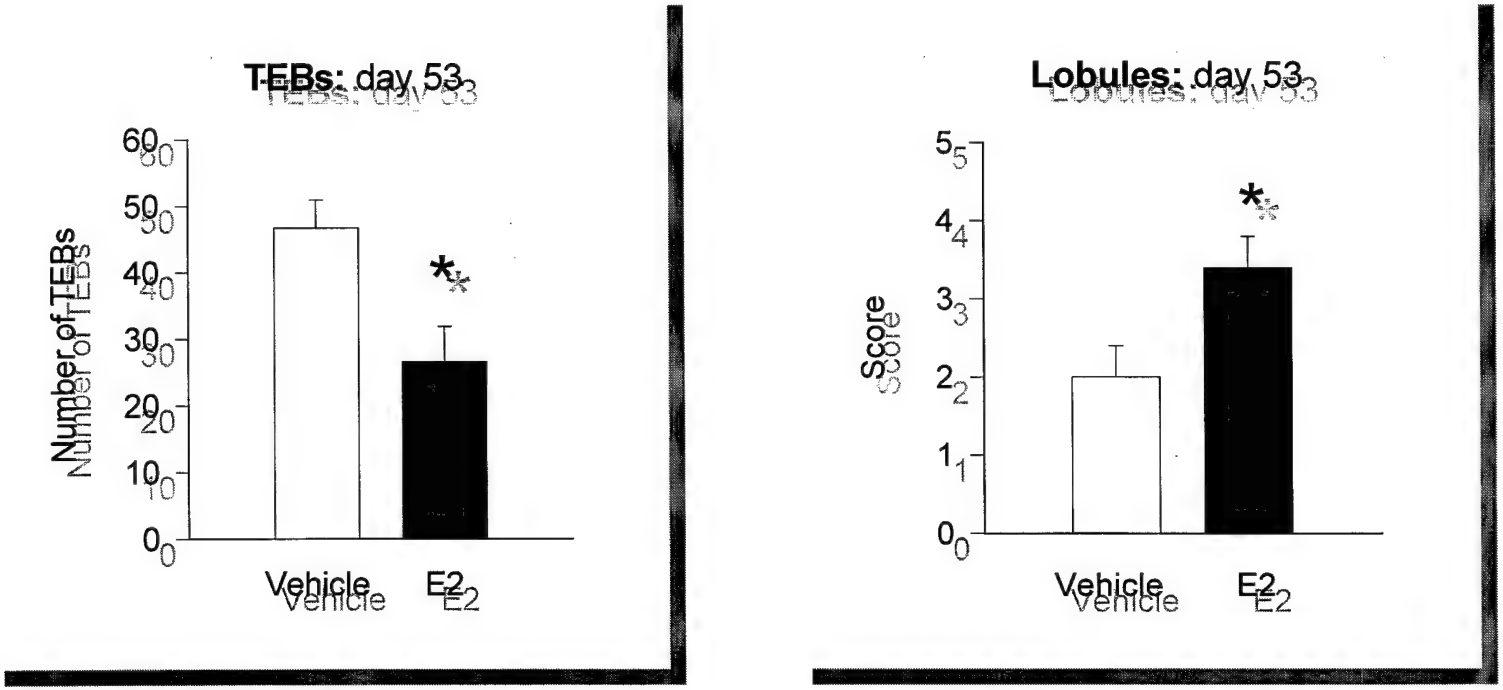
It critical to determine the mechanisms mediating the effects of *in utero* or prepubertal estrogenic exposures on mammary tumorigenesis. We have found that *in utero* exposure to either estradiol, high n-6 PUFA or genistein alters normal mammary gland development [9]. The glands exposed to estrogenic compounds *in utero* contain persistent terminal end buds (TEBs) and/or exhibit reduced differentiation to lobuloalveolar units (LAUs). TEBs play a central role in mammary gland development. They are the most actively growing epithelial structures, and contain cap cells which are interpreted to represent a pluripotent stem cell population. These cap cells are located on the basal surface of the TEB beneath the basal lamina. TEBs are known to be the sites of malignant transformation in the rodent mammary gland, and possibly also in the human breast. These data show that perinatal exposure to estrogenic compounds can alter mammary gland development, which in turn might be associated with increased susceptibility to develop breast cancer.

In contrast to delayed and/or reduced mammary differentiation in animals exposed to high *in utero* environment, we have noted that prepubertal estrogen exposure accelerates mammary epithelial differentiation **(Fig. 4)** [2]. Further, a prepubertal exposure to genistein causes changes in the mammary gland [3]. Similarly than in the prepubertally estradiol exposed rats, these changes can be characterized as increased differentiation of TEBs to LAUs. The differentiated mammary gland exhibits low or no susceptibility to carcinogen-induced malignancies, while non-differentiated gland containing high levels of TEBs is particularly prone to develop malignancies, if exposed to, for example, carcinogens.

Under Task-2, we are identifying epigenetic changes in the mammary glands of animals exposed to estrogenic manipulations during *in utero* or prepubertal period. These changes focus on two systems:

Figure 4.

PREPUBERTAL E2 EXPOSURE AND MAMMARY GLAND MORPHOLOGY



Mammary whole mounts of rats exposed to E2 during prepuberty contained significantly **fewer** terminal end buds and **more** lobules than those of vehicle-exposed rats.

(i) COX enzymes that convert arachidonic acid to prostaglandins, and (ii) the estrogen receptor.

2.1. Eicosanoids

No data to determine changes in COX-1 or COX-2 expression in the mammary glands of animals exposed to high or low levels of n-6 or n-3 PUFA *in utero* through a maternal diet or prepuberty, have been yet generated. These experiments will be started in January 2001, and they will be done by Susan Olivo, a graduate student in my laboratory. She has previously measured COX-1 and COX-2 expression in the rat colon, as part of her M.A. thesis.

2.2. ER-α and ER-β

We have established assays to determine both the protein and mRNA expression levels of ER-α and ER-β in the rat mammary gland **(Figs. 5 and 6)** and in vitro in human breast cancer cell lines. So far, we have determined these levels in the mammary glands of rats exposed to estradiol during prepuberty (results reported below). We are also currently determining ER-α and ER-β protein levels in the mammary glands of rats which were exposed to high or low n-6 PUFA diets either *in utero* or during prepubertal period.

In rats exposed to estradiol during prepuberty, the ER-α and ER-ß protein levels were determined at two different stages: in the mammary glands of non-carcinogen treated rats at the age of 53 days, and also in the mammary glands of 6-month-old rats that had been treated with DMBA 17 weeks earlier. ER-α antibody (MC-20; Santa Cruz Biotechnology, Inc) recognized a single immunoreactive band at 61 kDa. Preabsorbing the antibody with excess of the blocking peptide completely blocked the immunodetection of ER-α in the mammary gland.

We used a polyclonal antibody raised against the ligand binding domain of ER-β (amino acids 320-527) to determine ER-β protein levels (obtained from J-Å Gustafsson's laboratory, Karolinska Institutet, Sweden). This antibody recognizes rat, human and mouse ER-β, but not ER-α. We found that ER-β protein from the mammary gland migrated at approximately 62 kDa under our Western

blotting conditions, which is consistent with the size predicted from the first and/or second of the three possible ATG codon start sites on the ER-β sequence.

Figure 6 shows ER-α bands in one Western blot and **Figure 7** ER-β bands in another blot. Among the 6-9 mammary glands per group, no ER-α protein was detected in the 53-day old rats exposed to E2 during prepuberty, while tamoxifen-treated rats showed an increase in ER-α (3-fold) levels, compared to controls. Mammary ER-ß levels were increased by 4- fold in the E2-group and by 3.5-fold in the tamoxifen-group at the age of 53 days. These results are summarized in **Table 1**.

Table 1. Effects of prepubertal exposure to 10 µg estradiol (E2) or 100 µg tamoxifen on ER-α and ER-β protein levels. The results are expressed as a fold-difference, compared to the levels seen in the vehicle-control rats. N=3-9 rat mammary glands per treatment, receptor subtype, and age.

Fold-difference, compared to control glands.	ER-α 53-day-old rats, prior to DMBA	ER-β 53-day-old rats, prior to DMBA	ER-α Exposed to DMBA	ER-β Exposed to DMBA
Estradiol	No ER protein detected	4.0 ⇑	No change	2.5 ⇓
Tamoxifen	3.0 ⇑	3.5 ⇑	No change	No change

In the 6-month-old, DMBA-exposed rats, ER-α levels were not altered among the groups, which consisted of 3-6 rats each. ER-ß levels were 2.5-fold lower in the rats exposed to E2 at prepuberty than in the controls. ER-β levels in the DMBA-treated, prepubertally tamoxifen-exposed rats did not differ from those detected in the vehicle-control group.

Figure 5.

ER-α PROTEIN EXPRESSION IN RAT TISSUES

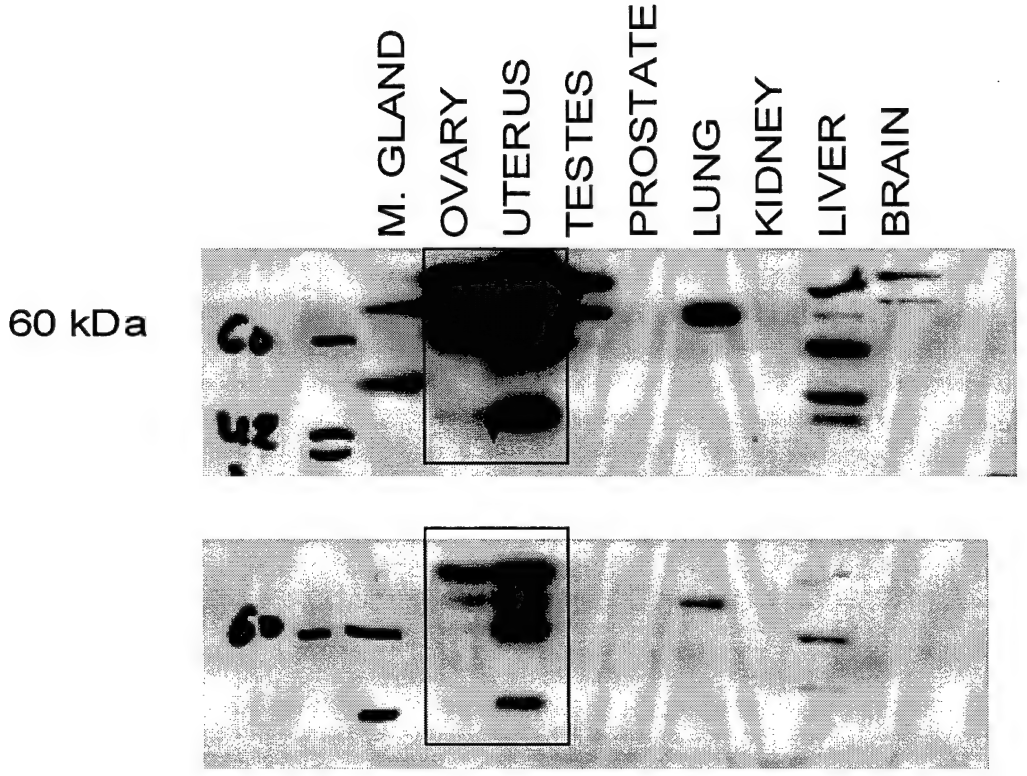
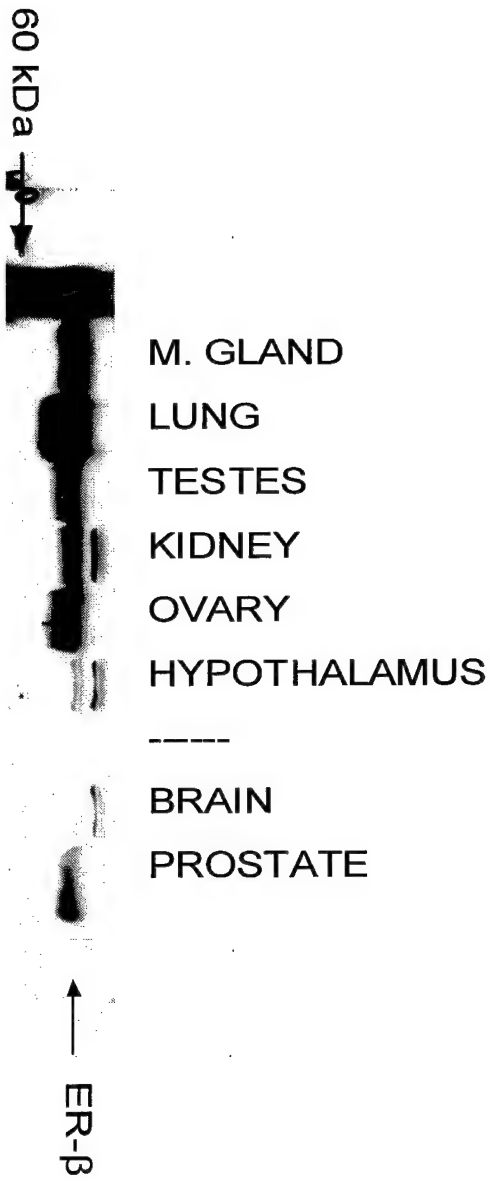


Figure 6.


ER-β PROTEIN EXPRESSION IN RAT TISSUES
60 kDa
M. GLAND
LUNG
TESTES
KIDNEY
OVARY
HYPOTHALAMUS

BRAIN
PROSTATE
ER-β

ER-α PROTEIN EXPRESSION IN MAMMARY GLAND

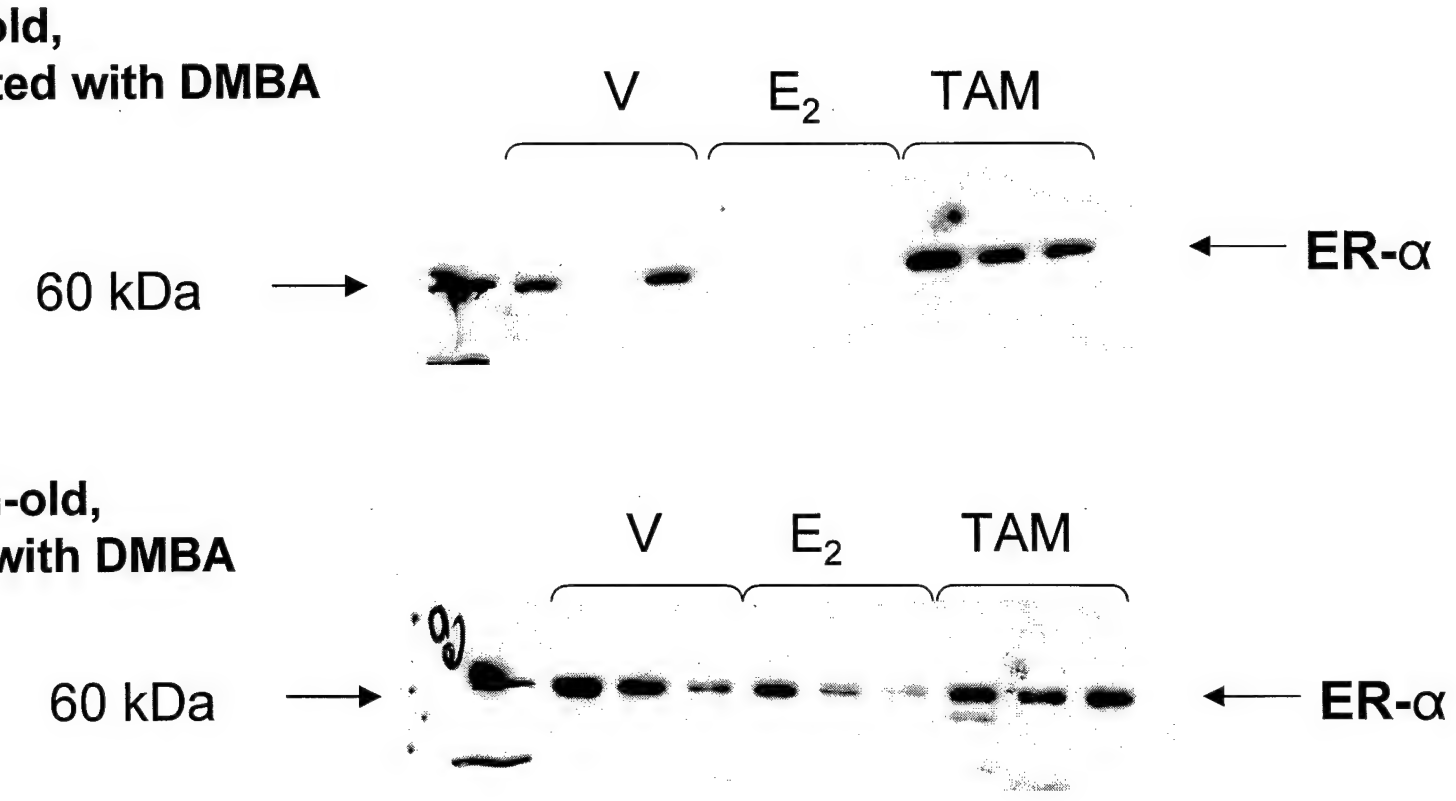
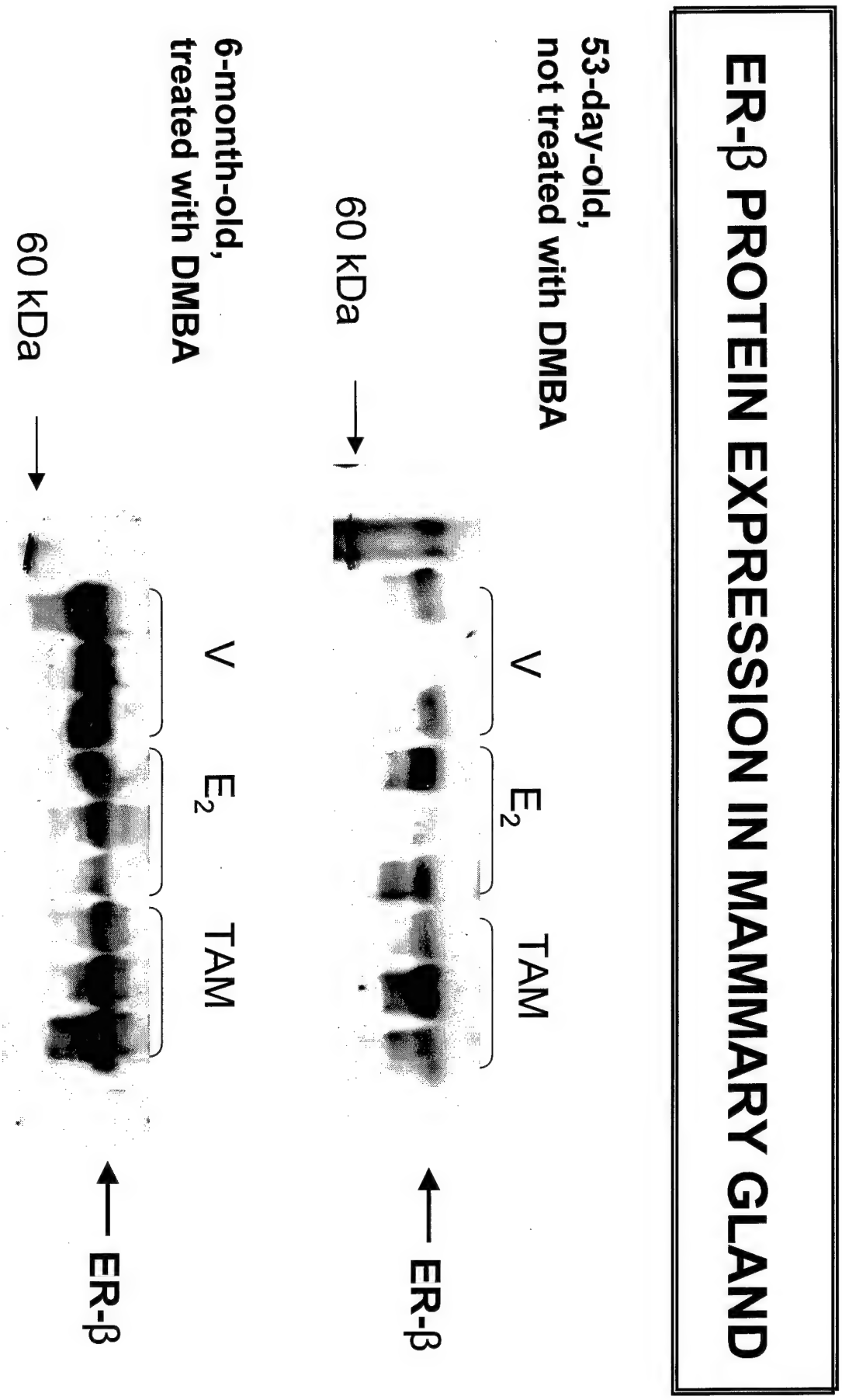


Figure 7

Figure 8.


ER-β PROTEIN EXPRESSION IN MAMMARY GLAND
53-day-old, not treated with DMBA
V
E2
TAM
60 kDa
ER-β
6-month-old, treated with DMBA
V
E2
TAM
60 kDa
ER-β

Task-3. Study whether (1) dietary fat intake and weight gain alter pregnancy estrogen levels in women, (2) affect possible intermediate biomarkers of increased breast cancer risk, as determined in nipple aspirate fluid, and (3) increase subsequent breast cancer risk (months 1-36)

3.1. The interactions among diet, weight gain and circulating estrogens in 200 pregnant women (months 1-18)
3.2. The effects of diet and weight gain on intermediate biomarkers in NAF (13-24)
3.3. Cohort study using >15,000 women to investigate whether pregnancy weight gain increases subsequent breast cancer risk. (24-36)

Research accomplished associated with Task-3

Differentiation reduces susceptibility to breast cancer, as is evident through findings showing that if pregnancy, which is characterized both by high estrogen levels and marked differentiation of the breast, occurs prior to age 20, breast cancer risk is reduced. However, first pregnancy that occurs after age 30 is associated with an increase in breast cancer. Further, pregnancy also induces a short-term increase in risk, lasting approximately 5 years after pregnancy in women who are 25 or older at first pregnancy. Within the first 12 months after pregnancy, risk to develop breast cancer may be increased by 20-fold. There is evidence to suggest that the higher pregnancy estrogenicity is, the higher is breast cancer risk. Thus, women with the highest range of pregnancy estrogens, such as those who took diethylstilbestrol or suffered from severe nausea, exhibit increased breast cancer risk, while women with low pregnancy estrogens who suffered from hypertension/pre-eclampsia, exhibit reduced breast cancer risk. Estrogen levels are approximately 10 times higher in pregnant than in non-pregnant women, but the levels also exhibit a marked inter-individual variability (4-6 fold) during pregnancy. **It is critical to identify factors that contribute to the normal inter-individual variability in pregnancy estrogen levels.** Diet, particularly dietary fats, may affect pregnancy estrogen levels. Data obtained in women indicate that obesity is associated with high circulating estrogen levels, while low fat intake reduces circulating estrogens. As indicated above, consumption of a high fat diet, containing mostly n-6 PUFA, significantly increased pregnancy estrogen levels. The data further showed that female rats kept on a high fat diet during pregnancy subsequently exhibited a significantly increased mammary tumor incidence, when compared with dams kept on a low fat diet during pregnancy.

It is also critical to identify those factors that mediate the effects of high estrogen and/or high fat diet during pregnancy on breast cancer risk. The mechanisms by which high pregnancy estrogen levels might increase breast cancer risk is by inducing proliferation of "initiated" cells, i.e., cells that have already undergone the first steps of neoplastic transformation. As a consequence of being activated by estrogens, neoplastic cells are likely to begin to secrete growth factors that further stimulate their growth. These growth factors possibly include epidermal growth factor (EGF), transforming growth factor (TGF-α), and insulin like growth factor 1 (IGF-1). We therefore propose that increased levels of these growth factors following pregnancy indicate that a woman is at an increased risk to develop breast cancer. We further propose that these growth factors should be determined from the nipple aspirate fluid (NAF) that reflects the immediate environment of the breast, rather than from the serum. NAF is secreted continuously by the non-lactating breast, and it can be obtained through aspiration of the nipple with a breast pump similar to that used to pump breast milk.

Our proposed study has three general aims: **(1)** to study whether diet, and dietary fat intake in particular, affects pregnancy estrogen levels in women, **(2)** to study whether a persistent change in possible intermediate biomarkers of increased breast cancer risk occurs in NAF from women who exhibited the highest circulating estrogen levels during pregnancy. The presence of hyperplastic epithelial cells also will be assessed, and **(3)** to study whether markers of high or low pregnancy estrogen levels are linked to subsequent breast cancer risk.

The two first aims will be studied in 200 pregnant women attending the Maternity Clinic at Solna in Stockholm, Sweden. First, the associations among serum E2 levels, diet, and weight gain in these pregnant women on gestation weeks 12, 22, and 32 will be investigated. Then, biomarkers of increased risk will be studied in two groups of women (n=50 in each): those who exhibited the highest quartile of circulating E2, and those who exhibited the lowest quartile of E2 during pregnancy. These biomarkers will be determined in NAF. **No studies to date have compared growth factor levels in NAF from women with no breast abnormalities, but who may be at an increased risk to develop breast cancer due to high circulating pregnancy estrogen levels.** The third aim will be studied using a cohort of 17,416 Finnish women. Weight gain will be used as a surrogate marker of high pregnancy estrogen levels. Within this cohort, we will identify women who developed breast cancer within 5 years following their last pregnancy. Pregnancy weight gain will

then be compared between the cases and appropriately matched controls.

3.1. The interactions among diet, weight gain and circulating estrogens in 200 pregnant women.

After the grant was funded, a collaboration was established between the P.I. (Leena Hilakivi-Clarke) and Professor **Hans-Olov Adami's** group in Sweden. Dr. Hans-Olov Adami is a Professor of Cancer Epidemiology at Karolinska Institute in Sweden. He is an author of about 450 scientific publications and has considerable expertise in clinical and epidemiological studies in the field of timing of estrogen exposure and breast cancer. Recently, he participated in a study in Sweden that involved recruiting pregnant women. The group successfully recruited 92% of eligible pregnant women who were followed throughout the pregnancy. Of these women 95% provided repeated blood and urine samples. The investigator overseeing the fieldwork in Sweden is Dr. **Elisabete Weiderpass.**

All preparations to recruit 200 pregnant women in Sweden has been completed, and a mid-wife has been hired to collect blood samples, dietary intakes, and demographic information. The recruited women have to be 25 years old or older, since pregnancy does not increase breast cancer risk in women who are younger than 25, and have no pre-existing conditions that may affect pregnancy. Subjects with pre-existing diabetes mellitus, hypertension, or breast cancer are excluded. Smokers and heavy alcohol users also will be excluded. Information on the known risk factors of breast cancer (family history, history of benign breast disease, age at menarche, onset of menarche, and age at first birth) also will be collected. These factors will be obtained through a detailed demographic questionnaire. Women in the study will also attend the maternity clinic regularly. During weeks 12, 22, and 32 of pregnancy, body weight, body mass index, dietary intakes, and serum will be collected from the participants. *The main emphasis of the study is to determine whether pregnancy diet and weight gain affect pregnancy estrogen levels.*

200 pregnant women are estimated to have been recruited by the end of 2000, and by summer 2001 collection of their dietary records and serum estrogens are completed.

Statistical methods. Two hundred fifty women will be enrolled on study under the current protocol.

We estimate that at most 50 (20%) of them might be dropping out due to subsequent pregnancy complications, leaving a total sample size to 200 pregnant women. This sample size provides 87% power to detect a positive correlation greater than 0.3 with estradiol levels if the true correlation is 0.5 at a two-sided 2.5% significance level, for dietary fat intake, serum fatty acids, pregnancy weight gain, and body fat, separately. This power calculation has taken into account the reported mean and standard deviations for E2 during pregnancy, particularly during gestation weeks 12, 22, and 32. Multiple linear regression will be performed to determine the effect of weight gain, body fat, serum fatty acids and dietary components, such as the intake of n-6 and n-3 PUFAs, on total pregnancy estradiol levels on pregnancy weeks 12, 22 and 32. The means of three 24 hr dietary records prior to each visit will be calculated, and used in comparisons to serum E2 levels. Thus, the association between dietary intakes and serum E2 levels for each of the three gestation weeks, will be done separately. We also will analyse whether weight gain and dietary factors differ in women that exhibit the highest versus lowest pregnancy estrogen levels, using appropriate parametric or non-parametric tests.

Caloric requirements are increased during pregnancy and metabolic efficiency may also change. Intake of specific nutrients is strongly correlated with total caloric intake. Therefore, the multiple linear regression to examine associations between dietary fat intake (and other nutrients) and serum E2 will use calorie adjusted nutrient intakes obtained using the regression approach described by Willett and Stampfer. This adjustment provides estimates of dietary fat (or other nutrient) intake that are independent of total caloric intake. The calorie adjusted nutrient estimates are obtained by regressing each nutrient on caloric intake (in separate regression models), then using the regression residuals (plus a suitable constant) in place of each woman's absolute nutrient intake as independent variables in the multiple linear regression on serum E2.

Taking account of errors in the estimation of fat intake. Correlation is a function of the variability of two measures. This variability includes natural variation from person to person, variation within one person from day to day, and measurement error. There is measurement error for every factor we will analyze. The measurement error associated with fat intake may be larger than some others but it is likely to be uniformly low and will not substantially interfere with a test of correlation with other factors. Since the study has substantially high power (87%) to detect a

correlation greater than .3 at a stringent two-sided significance level (2.5%), the variability added by error in calculating fat intake is not expected to pose any danger to the results of this study.

3.2. The effects of diet and weight gain on intermediate biomarkers in NAF

We are planning to collect nipple aspirate fluids from 50 women who participated to being tested for Hypothesis-1 and who had the highest pregnancy E2 levels, and also from 50 women who had the lowest pregnancy E2 levels. Additional criteria are that these women were nursing at least for 3 months but not longer than 7 months. We expect that most women have stopped nursing at this point, because the average length of nursing in Sweden is 6 months. Since most of Swedish women breast feed, we do not anticipate any difficulties in obtaining 50 women to each of the categories. In contrast, it is not possible to obtain 50 women exhibiting high and 50 women exhibiting low pregnancy estrogen levels who are not nursing at all among the pool of 200 women. Therefore, we are not planning to compare NAF growth factor levels between nursing and non-nursing women. The average of the three E2 measurements on gestation weeks 12, 22, and 32 will be used as an index of total E2 exposure during pregnancy. Since the inter-individual circulating E2 levels are expected to vary 4-6 fold, the values being relatively evenly distributed across the range, we do not expect that there is any overlap between the lowest E2 values in the "high" E2 group and the highest values in the "low" E2 group. As indicated above, E2 levels on the eight month of pregnancy for example may vary from as low as approximately 10 ng/ml to as high as 50 ng/ml.

Experimental Protocol. Collection of NAF and other measurements will be performed approximately 8 months after giving birth and at least 1 month after stopping to nurse. As indicated above, we have a strong reason to believe that if a woman has initiated cells in her breast, growth factor levels are increased and keep increasing parallel to the growth of initiated/malignant cells, at least for 12 months after pregnancy. We are not planning to obtain NAF from women who nursed longer than 7 months, because it is possible that long nursing counteracts some of the effects of pregnancy estrogenicity on breast cancer risk. This intriguing possibility will be addressed in our future studies.

We hypothesize that biomarkers in NAF are increased in women who are at an increased risk to develop breast cancer, reflecting the fact that high pregnancy estrogenicity stimulated the growth of an existing malignancy in the breast. The levels of biomarkers should consistently increase over time, paralleling an increase in tumor growth. Although menstrual cycle does not appear to affect NAF levels of E2, IGF/IGFBP-3, EGF, or TGF-α, we will control for the stage of the cycle. We also will measure serum E2 levels at the time NAF is collected. Thus, NAF and serum will be collected on the late luteal phase (day 20-24 after the start of last menses), since estrogen levels are then higher than during follicular phase, but lower than during ovulation estrogen peak.

To investigate whether NAF biomarker levels exhibit an inter-individual consistency, we ask women to come to a second NAF collection 1 month after the first collection is performed. We then compare the levels of E2, IGF/IGFBP-3, EGF, or TGF-α in NAF obtained during these two separate sessions.

We estimate that **nipple aspirate fluids** will be obtained during summer and fall of 2001. They will be analysed by March 2002, after which the final statistical analysis and preparation of the manuscript will be done.

3.3. Cohort study using >15,000 women to investigate whether pregnancy weight gain increases subsequent breast cancer risk.

A cohort of 17,416 Finnish women will be used to determine whether pregnancy estrogenicity is linked to subsequent breast cancer risk. No blood was collected, and therefore we will use indirect indicators of high pregnancy estrogenicity, including pregnancy weight gain. The study will be done in collaboration with Dr. **Riitta Luoto**, MD/PhD, a Finnish Epidemiologist at the University of Tampere. The study is part of a surveillance program for women using intrauterine hormone-releasing device called Levonova (Mirena^{R}). The women who had Levonova inserted in the time period from April 1990 to December 1993 were mailed a questionnaire. This questionnaire consisted of 75 separate items. Two questionnaires were sent to each woman. One questionnaire was intended to Levonova user and the other was asked to be given to a sister or friend in order to gather a

reference group of women not using hormonal contraception. The questionnaire contained items regarding general health status, sociodemographic background, reproductive and contraceptive history, and gynaecological problems. In addition, information on the amount of weight gained during pregnancy and whether a woman developed breast cancer after pregnancy, were acquired through the questionnaire. The final number of completed questionnaires available is 17,416.

Within this cohort, we will identify those women who were diagnosed with breast cancer 0-5 years after the last pregnancy, through files kept by the Finnish Cancer Registry (cases). All (100%) newly diagnosed malignant tumors in Finland will be reported to the National Finnish Cancer Registry. Controls will be women, also in the cohort, that have never been diagnosed with cancer (controls). The controls will be matched with the cases in terms of age, the age at pregnancy when the reported weight gain occurred, and the contraceptive status. Possible differences in pregnancy weight gain will then be compared between the cases and controls. In addition to studying whether pregnancy weight gain increases breast cancer risk within 5 years of the last pregnancy, the cohort also can be used to study the effect of hormonal intervention after pregnancy on breast cancer risk, in women exhibiting high weight gain during pregnancy.

Statistical Methods used in the analysis. The study design is that of a nested cohort study in which the initial cohort is a group of women who had a progestin-releasing intrauterine device inserted following their last pregnancy as well as women who did not use this birth control method or any other hormonal birth control method. Among this cohort is a group of women with breast cancer first diagnosed within 5 years following last pregnancy. The control (a woman who has never been diagnosed with cancer) for each case is a woman who is the same age, had her last child at the same time as the case (± 1 year), and had IUD inserted (if the case also had) or not. When the cases and their controls have been identified, pregnancy weight gain for both groups will be determined. We also will analyse separately women with the hormonal intrauterine device from those that do not have it. The cohort contains 17,416 premenopausal women. Based on age-specific incidence ratesin Finland, as reported by the Finnish Cancer Registry, **we estimate that approximately 100-150 women (20-30 women annually) in the cohort have a diagnosis of breast cancer within a 5 year period following pregnancy.**

The data analysis will be done in Finland. We have identified a statistician who will be performing initial analysis of the data during this summer (2000).

3.4. Additional achievements related to Task-3.

We have recently completed a project that is directly related to Task-3, and funding is being applied to an other project also related to this Task.

3.4.1. TALLNESS AND OVERWEIGHT DURING CHILDHOOD HAVE OPPOSING EFFECTS ON BREAST CANCER RISK

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T Forsén[2], MD, PhD	Research Fellow
JG Eriksson[2], MD, PhD	Senior Researcher
R Luoto[3], MD, PhD	Senior Researcher
J Tuomilehto[2], MD, PhD	Professor
C Osmond[4], PhD	Statistician
DJP Barker[4], MD, PhD	Director

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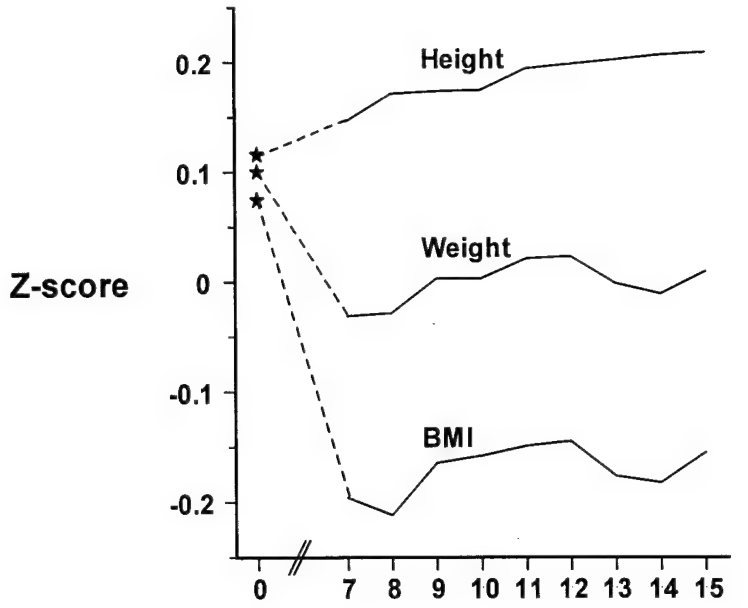
Abstract

Background Body size in adult life is associated with breast cancer risk. Pre-menopausal women who have low body mass are at increased risk of developing the disease. After the

EARLY BMI, HEIGHT AND BREAST CANCER RISK



Height and body mass index during childhood in women who later developed breast cancer.

- **High BMI (high estrogenicity) reduces risk.**
- **Tallness (low estrogenicity) increases risk.**

Figure 9.

menopause, greater body weight and tallness increase the risk. We studied how weight and height during childhood affect breast cancer risk.

Methods Birth and school health records containing information on maternal, neonatal, and childhood measurements of body size were obtained for 3,447 women born during 1924-33 at the University Hospital of Helsinki, Finland. Through linkages with the National Hospital Discharge Registry and the Cause of Death Registry women who developed breast cancer during 1971 - 1995 were identified. We found that 177 women had been admitted to hospital with breast cancer, of whom 49 had died from the disease. 135 (76%) of these women were aged 50 years or more at the time of diagnosis, and were therefore likely to have been post-menopausal. We examined the trends in hazard ratios for breast cancer with measurements of body size at birth and during childhood.

Results Hazard ratios for breast cancer rose with increasing weight and length at birth, though neither trend was statistically significant. At each age, from 7 to 15 years, the girls who later developed breast cancer were on average taller and had lower body mass than the other girls **(Fig. 9).** Unadjusted hazard ratios rose across the range of height ($p = 0.01$ at age 7 years) and fell across the range of body mass index ($p = 0.009$ at age 7 years). In a simultaneous analysis the unadjusted hazard ratio for breast cancer was 1.31 (95% CI 0.97 – 1.78, $p = 0.08$) for every kilogram increase in birth weight and 1.21 (95% CI 1.06 – 1.38, $p = 0.004$) for every kg/m^2 decrease in body mass index at 7 years.

Conclusions Tallness in childhood is associated with increased risk of developing breast cancer. One possible explanation is that tall childhood stature reflects high plasma concentrations of insulin-like growth factors or low estrogen levels which, persisting throughout life, may result in an increased vulnerability to breast cancer. In contrast, being slightly overweight in childhood reduces breast cancer risk. The increased adipose tissue -derived oestrogen levels in overweight children could induce early breast differentiation and eliminate some undifferentiated mammary epithelial cells as targets for malignant transformation.

3.4.2. *IN UTERO* ESTROGENICITY AND INDICATORS OF BREAST CANCER RISK

DURING PUBERTY

The project group is based on a collaborative effort that has been established between Dr. Dimitrios Trichopoulos and The Center for Cancer Prevention at Harvard School of Public Health (HSPH), and Dr. Lars Vatten and the Medical School at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway. Dr. Leena Hilakivi-Clarke at the Vince Lombardi Cancer Center at Georgetown University, Washington DC has joined this collaboration.

SPECIFIC AIMS

Estrogens may have different effects on breast cancer risk, depending on the timing of exposure. Fetal exposure to high *in utero* estrogenicity may increase the risk of breast cancer in adult life, but estrogen exposure during adolescence appears to reduce the risk. High fetal estrogenicity may be associated with high birth weight and with perimenarcheal tallness, but it has been shown that exposure to high intrauterine estrogenicity may not necessarily be followed by high estrogenicity during childhood. Animal studies have shown that high fetal estrogenicity is associated with early puberty onset, but this has not been studied in humans. Overall, *in utero* estrogencity appears to be positively associated with birth weight, childhood and adolescent tallness, and with early age at menarche. Since all these factors are associated with the subsequent risk of developing breast cancer, it is conceivable that high fetal estrogenicity may induce a tracking pattern that may accumulate into a higher breast cancer risk.

It may seem paradoxical that high *in utero* estrogenicity may be associated with reduced, rather than increased estrogenicity in childhood, since this appears to contradict the hypothesis that low estrogen levels reduce breast cancer risk. However, both human and animal studies have shown that high estrogenicity in childhood/adolescence may actually reduce breast cancer risk, supporting the hypothesis that during this period in life, high estrogen levels may induce effects on the breast that are protective against cancer. Low childhood estrogenicity is also associated with other factors that are related to developing breast cancer. These factors include insulin like growth factors (IGFs) and their binding proteins, since high estrogen levels reduce IGF levels at puberty, and IGFs in turn play an important role in determining stature and the onset of menarche.

In this study, we propose to examine whether a longitudinal pattern may be recognized between

potential risk factors for breast cancer that operate during very early life (*in utero* and perinatal) and adolescent risk factors. In a prospective study of Norwegian women who were closely followed during pregnancy, and their female offspring, who are presently about to enter puberty, we propose to examine associations between maternal influences (circulating estrogen and IGF-1 levels, and pre-eclampsia) that were registered during pregnancy and perinatal factors (birth weight, placental weight, infant jaundice, and factors measured in umbilical cord blood) that were registered in the female newborns at birth. In a follow-up at adolescence of these girls, we now propose to collect information on physical growth, pubertal development, nutrition, and to measure estrogen and IGF levels. We want to emphasize that to be successful, this study needs to be performed in the immediate future, since this cohort of young girls is presently entering adolescence.

Our specific aims consist of the following research questions:

Does high *in utero* estrogenicity induce early menarche?

Is high birthweight an intermediate marker between high *in utero* estrogenicity and early menarche?

What are the critical differences in girls who (a) were exposed to high *in utero* estrogenicity, are tall and enter menarche relatively early (increasing breast cancer risk), and girls who (b) were exposed to high estrogenicity in childhood, enter menarche early, but remain relatively short (reducing breast cancer risk) ?

Which factors influence stature at the time of puberty, and before and after puberty?

To meet these objectives, we present the following hypotheses:

Hypothesis-1. High intrauterine estrogenicity will advance the onset of puberty, and stimulate somatic growth, including height, at adolescence. In contrast, indicators of low intrauterine estrogenicity will delay the onset of puberty, and slow down somatic growth.

Hypothesis-2. High birth weight is associated with high intrauterine estrogenicity and early menarche; i.e., high birth weight is an intermediate marker of early menarche. Other indicators of high intrauterine estrogenicity, including high placental weight and infant jaundice, will also advance puberty onset, while indicators of low estrogenicity, including pre-eclampsia, will delay puberty.

Hypothesis-3. Indicators of high intrauterine estrogenicity are associated with high IGF-I levels at adolescence, and we propose that IGF-I may be associated with early onset of menarche and with increased stature. In contrast, high BMI in childhood and at puberty is associated with increased estrogenicity during this period, and shorter postpubertal stature.

This study will provide new information on how the intrauterine environment may initiate a possible tracking pattern between early estrogenic influences and the somatic and reproductive development of young girls during their perimenarcheal period, as determined by clinical characteristics and hormone and growth factor levels during adolescence. Ultimately, a tracking pattern starting during the intrauterine period and ranging throughout childhood and adolescence may play a critical role in understanding the risk of developing breast cancer.

Task-4. Development of Course in Nutrition and Breast Cancer (months 12-18)
4.1. Outline the course
4.2. Integration of the course to the existing Tumor Biology Course

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Research accomplished associated with Task-4.

4.1. Outline the course.

Attached is a completed outline of the course entitled "**Life-style and Cancer Prevention**"

4.2. Integration of the course to the existing Tumor Biology Course.

This course will be first taught for Tumor biology students beginning in Spring Semester 2001. All preliminary preparations for the course, including speaker selection and confirmation has been completed.

Tumor Biology Program - Life-style and Cancer Prevention

Spring, 2001

Offered by the Tumor Biology Graduate Program

Meeting Place:

Day & Time:

Format: The course will consist of presentations primarily by the Faculty and class discussions of any materials provided to the students in advance. Students may be required to read up to 4 papers per week, and to participate in all class discussions.

This is a two credit, advanced course in Cancer Prevention. The course will cover the life-style related risk factors associated with selected cancers with special emphasis on the nutrition, environment, and specific behaviors.

The goals of the course are to (1) provide students with an understanding of the general principles involved in cancer prevention by life-style modifications from both the basic science, clinical and epidemiologic perspectives, (2) develop critical scientific reading and comprehension skills, and to (3) develop interactive skills within a scientific discussion forum.

Grading: Mean grade from a mid-term exam and final library research paper. For the latter, subjects and mentor to be chosen by students from within course Faculty.

Course Directors: Leena Hilakivi-Clarke, PhD, Associate Professor of Oncology, and Marc Schwartz, PhD, Assistant Professor.

Information: Leena Hilakivi-Clarke
Lombardi Cancer Center
W405A New Research Building
Tel: 687-7237

TUMOR BIOLOGY PROGRAM - LIFE STYLE AND CANCER PREVENTION

Part 1: Basic Concepts

1. Introduction to the Course	Hilakivi-Clarke
2. Basic Concepts of Nutrition	Storey
3. Basic Concepts of Behavioral Science	Schwartz
4. Basic Concepts of Chemical Carcinogenesis	Shields
5. Basic Concepts of Cancer Epidemiology	Trock

Part 2: Nutrition and cancer risk

1. Dietary Fats	Hilakivi-Clarke
2. Calories and Obesity	Storey
3. Phytoestrogens	Hilakivi-Clarke
4. Other Phytochemicals	Clarke
5. Vitamins	Byers
6. Fiber	Storey
7. Alcohol	Hilakivi-Clarke

Part 3: Other life-style factors and cancer risk

1. Smoking and Cancer Risk	Lerman/Audrain
2. Environmental Pollutants and Cancer Risk	Trock
3. Heavy Metals and Cancer Risk	Martin
4. Physical Activity and Cancer	Audrain
5. Psychosocial Factors and Cancer Risk	Hilakivi-Clarke

Part 4: Cancer risk and behavior

1. Life-style Interventions	Hughes/Audrian
2. Cancer Risk and Psychosocial Factors	Schwartz
3. Early Detection	Taylor
4. Behavioral Aspects of Genetic Testing for Cancer Susceptibility	Schwartz
5. Sociocultural Aspects of Cancer Prevention and Control	Hughes

Grading

Mid-term exam: A written exam on parts 1 and 2.

KEY RESEARCH ACCOMPLISHMENTS

- timing of estrogen exposure determines whether this hormone increases, decreases or has no effect on breast cancer risk.
  - *in utero* estrogen exposure increases risk.
  - prepubertal estrogen exposure decreases risk.
- timing of an exposure to dietary components that influence estrogenicity (PUFAs or phytoestrogens) has a similar impact than an exposure to E2, perhaps also in women.
- increased expression of ER-β protein may protect the mammary gland from malignant transformation.
- establishment of collaborations with epidemiologists in Sweden (Drs. Adami and Weiderpass), Norway (Dr. Vatten), Finland (Drs. Luoto, Eriksson and Koskenvuo), United Kingdom (Dr. Barker) and US (Dr. Trichopoulos) to study the importance of timing of estrogenic/dietary exposures in affecting breast cancer risk in human populations.
- development of a course to teach aspects relating to diet and cancer.

REPORTABLE OUTCOMES

Publications 1999 -

1. HILAKIVI-CLARKE LA, CLARKE R, LIPPMAN M. The influence of maternal diet on breast cancer risk among female offspring. *Nutrition* 15:392-401, 1999.

2. HILAKIVI-CLARKE LA, ONOJAFE I, RAYGADA M, CLARKE R. Maternal genistein exposure during pregnancy increases breast cancer risk among female offspring. *Oncology Reports* 6:1089-1095, 1999.

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4. CLARKE R, SKAAR T, EL-ASHRY D, LEONESSA F, HILAKIVI-CLARKE LA. Use of

ERE and reported gene constructs to assess putative estrogenic activity. *J Medical Food*, in press 1999.

5. HILAKIVI-CLARKE LA, CHO E, ONOJAFE I, LIAO DJ, CLARKE R. *In utero* exposure to tamoxifen increases DMBA-induced mammary tumorigenesis. *Clinical Cancer Research*, in press, 2000.

6. CLARKE R, HILAKIVI-CLARKE LA, TROCK B. Dietary and environmental sources of estrogenicity and breast cancer risk. *Biologist,* in press 1999.

7. BOUKER K,HILAKIVI-CLARKE LA. Genistein: A powerful weapon in the breast cancer prevention arsenal or a potent mitogen? *Environmental Health Perspectives*, in press 2000.

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9. CABANES A, DE ASSIS S, GUSTAFSSON J-A, HILAKIVI-CLARKE L, . Maternal high-fat intake during pregnancy increases voluntary alcohol intake and hypothalamic estrogen receptor protein levels among female offspring. *Developmental Neuroscience*, in press 2000.

10. HILAKIVI-CLARKE L, CHO E, DEASSIS S, OLIVO S, EALLEY E, BOUKER KB, WELCH J, KHAN G, CLARKE R, CABANES A. Maternal and prepubertal diet, mammary development, and breast cancer risk. J Nutr, in press 2000.

11. HILAKIVI-CLARKE LA, FORSEN T, LUOTO R,ERIKSSON J, OSMOND C, BARKER D. Tallness and overweight during childhood have opposing effects on breast cancer risk. Submitted, 2000.

12. CABANES A, OLIVO S, DE ASSIS S, GUSTAFSSON J-A, HILAKIVI-CLARKE L Prepubertal estradiol exposure increases estrogen receptor beta levels in the mammary gland and reduces 7,12-dimethylbenz[a]-antracene-induced mammary tumorigenesis in rats.

Submitted, 2000.

Manuscripts in preparation

13. WHITE L, HILAKIVI-CLARKE L, CLARKE R, TROCK B. Meta-analysis of soy intake and breast cancer risk. A manuscript in preparation, 2000.

14. CABANES A, DEASSIS S, LIAO J, GUSTAFSSON J-A, HELFRICH W, HILAKIVI-CLARKE L. Soy diet during pregnancy reduces carcinogen-induced mammary tumorigenesis and causes a persistent increase in estrogen receptor β protein levels in the rat mammary gland. A manuscript in preparation, 2000.

15. CABANES A, DEASSIS S, LIAO J, GUSTAFSSON J-A, HELFRICH W, HILAKIVI-CLARKE L. A high maternal soy diet during pregnancy does not affect carcinogen-induced mammary tumorigenesis among female rat offspring A manuscript in preparation, 2000.

Abstracts

HILAKIVI-CLARKE, LA, CHO, E, MARTIN MB, CLARKE R. Maternal exposure to environmental estrogens during pregancy alters puberty onset, mammary gland development, and breast cancer risk among offspring. In: Endocrine Disruptors (b5). KEYSTONE SYMPOSIA on Molecular & Cellular Biology, 1/31-2/5/99. Tahoe City, California.

CHO E, ONOJAFE I, HILAKIVI-CLARKE L. A maternal exposure to high n-3 fatty acid diet during pregnancy and breast cancer risk among female rat offspring. 90th Annual Meeting, American Association for Cancer Research, April 10-14, 1999. Abstract 2400.

TROCK B, WHITE BUTLER L, CLARKE R, HILAKIVI-CLARKE L. Meta-analysis of soy intake

and breast cancer risk. 9th Annual Research Conference, American Institute for Cancer Research, September 2-3, 1999. Washington, DC.

HILAKIVI-CLARKE L. Growth during childhood and subsequent breast cancer risk. Susan G. Komen Breast Cancer Foundation, National Grants Conference, October 3, 1999. Dallas, Texas.

TROCK B, WHITE BUTLER L, CLARKE R, HILAKIVI-CLARKE L. Meta-analysis of soy intake and breast cancer risk. 3rd International Symposium of the role of soy in Preventing and Treating Chronic Disease. November 1999. Washington, DC.

HILAKIVI-CLARKE L, FORSEN T, ERIKSSON J, LUOTO R, BARKER D. Growth during childhood and subsequent breast cancer risk. American Association for Cancer Research Meeting, San Francisco, April 2000.

CABANES A, DEASSIS S, OLIVO S, GUSTAFSSON J-Å, AND HILAKIVI-CLARKE L. Prepubertal exposure to estradiol increases estrogen receptor beta levels in the developing mammary gland and reduces mammary tumorigenesis in rats. American Association for Cancer Research Meeting, San Francisco, April 2000.

Invited Presentations

1999 Workshop on Early Life Exposures and Risk of Breast Cancer. Arranged by National Action Plan on Breast Cancer. A presentation entitled "*An Overview of an Animal Model of Early Exposures to Environmental Estrogens and Dietary Factors*". Chantilly, Virginia. January, 1999.

1999 49th Harden Conference. Arranged by the Biochemical Society. A presentation in the Forum "Infant Nutrition - Brain Development - Disease in Later Life" that was entitled "*Dietary fat intake during gestation in mice: brain composition and behaviour in offspring*". In Oxford, England. August

1999.

2000 Annual Conference of American Institute for Cancer Research. A symposium presentation in the session "Nutrition, Normal Development and Cancer Prevention" entitled "*Maternal and prepubertal diet, mammary development and breast cancer risk*". In Washington DC, September 2000.

2000 The Susan G. Komen *Reaching for the Cure ... Making A Difference* Mission Conference. Oral presentation entitled "*Timing of estrogen exposure and breast cancer risk*". In Washington DC, September 2000.

2000 The Third Cooper Institute Scientific Conference: Physical Activity and Cancer. A symposium presentation entitled "*Physical activity, stress adaptation - habituation, and cancer*". In Dallas, Texas. November 5-7, 2000.

Workshops/Symposium attended

1999 "*Phytoestrogens*", a Workshop arranged by National Institute of Aging, Phoenix, Arizona, May 1999.

1999 "*The Role of Tissue Architecture in the Development of Breast Cancer*", a Workshop arranged by National Action Plan on Breast Cancer, in Washington, DC, September 15-16, 1999.

CONCLUSIONS

Summary of the results

The data obtained during the first year of funding emphasize that estrogens have different effects on breast cancer risk, depending on the timing of exposure. Some dietary components have estrogenic effects. Our studies have shown that a maternal exposure to a high n-6 or n-3 PUFA diet increases pregnancy estrogen levels. Phytoestrogens, including genistein present in soy, appear to activate the estrogen receptor. Thus, a new variable, timing of exposure should also be taken into account when the effects of diet on the risk of developing breast cancer are being assessed. Our data further indicate that the mechanisms by which early life dietary exposures affect breast cancer risk are related to changes in the mammary gland differentiation and expression of ER-α and ER-β. In particular, increase in ER-β protein levels might protect the breast from malignant transformation. During the remaining 2 years, we will be doing further studies to determine the specific roles of the two estrogen receptors in the mammary gland. We also will attempt to identify the best means to prevent some breast cancer by dietary modifications during childhood and pregnancy.

"So what section"

Although diet is clearly associated with breast cancer risk, studies have failed to provide convincing evidence in favor of a particular dietary component in causing or preventing breast cancer. This failure is likely to have been caused by not having been taking into consideration the fact that timing of exposure is critical. An exposure to the same dietary component might have a different effect on breast cancer risk, if the exposure occurs *in utero* through a pregnant mother, during childhood, puberty, pregnancy, reproductive years, or postmenopause. For example, our recent results and the results of other investigators indicate that an exposure to a phytoestrogen genistein during fetal life and postmenopause might increase breast cancer risk, while an exposure during childhood may provide a permanent protection. By determining the impact of timing of various dietary components, we are more likely to be able to prevent some breast cancers than by assessing the interaction between diet shortly before diagnosis and breast cancer risk.

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6. HILAKIVI-CLARKE LA, CHO E, RAYGADA M, ONOJAFE I, CLARKE R, LIPPMAN ME. Breast cancer risk in rats fed a diet high in n-6 polyunsaturated fatty acids during pregnancy. *Journal of National Cancer Institute*, 88:1821-1827, 1996.
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APPENDICES

No appendices are included. None of the publications resulting from the work supported by USAMRMC has been published (references 1, 2, 4, 7, 9).